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Synthesis and Processing of the Transmembrane Envelope Protein of Equine Infectious Anemia Virus

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The transmembrane (TM) envelope protein of lentiviruses, including equine infectious anemia virus (EIAV), is significantly larger than that of other retroviruses and may extend in the C-terminal direction 100 to 200 amino acids beyond the TM domain. This size difference suggests a lentivirus-specific function for the long C-terminal extension. We have investigated the synthesis and processing of the EIAV TM protein by immune precipitation and immunoblotting experiments, by using several envelope-specific peptide antisera. We show that the TM protein in EIAV particles is cleaved by proteolysis to an N-terminal glycosylated 32- to 35-kilodalton (kDa) segment and a C-terminal nonglycosylated 20-kDa segment. The 20-kDa fragment was isolated from virus fractionated by high-pressure liquid chromatography, and its N-terminal amino acid sequence was determined for 13 residues. Together with the known nucleotide sequence, this fixes the cleavage site at a His-Leu bond located 240 amino acids from the N terminus of the TM protein. Since the 32- to 35-kDa fragment and the 20-kDa fragment are not detectable in infected cells, we assume that cleavage occurs in the virus particle and that the viral protease may be responsible. We have also found that some cells producing a tissue-culture-adapted strain of EIAV synthesize a truncated envelope precursor polyprotein. The point of truncation differs slightly in the two cases we have observed but lies just downstream from the membrane-spanning domain, close to the cleavage point described above. In one case, virus producing the truncated envelope protein appeared to be much more infectious than virus producing the full-size protein, suggesting that host cell factors can select for virus on the basis of the C-terminal domain of the TM protein.

Equine infectious anemia virus (EIAV) is a lentivirus, related in genome organization (23, 36, 38), sequence (6, 23, 36, 38), and morphology (12) to the human immunodeficiency virus (HIV), the cause of acquired immunodeficiency syndrome. Depending on the host and the virus strain, an EIAV-infected horse may (i) suffer a brief acute illness and die, (ii) undergo multiple cycles of illness and recovery, or (iii) remain clinically normal. Acute episodes involve fever, viremia, weight loss, and leucopenia; and animals who have multiple episodes become severely anemic and may show central nervous system impairment. As with HIV, EIAV persists even in clinically normal individuals, and blood cells have been found to be infectious after disease-free intervals of many years (7, 22). The principal target cell is a leukocyte, probably the macrophage (22, 30, 31, 35).

Retrovirus transmembrane (TM) envelope proteins share a number of common features (for a review, see reference 11 and E. Hunter and R. Swanstrom, Curr. Top. Microbiol. Immunol., in press). They all have two highly hydrophobic regions separated by a 110- to 160-amino-acid stretch that contains at least two cysteine residues. One of these regions is at or near the N terminus and is thought to play a role in fusing the viral envelope with that of the host cell. The second hydrophobic stretch is the presumptive membrane-spanning region. Most retrovirus TM proteins, including those of type B, C, and D viruses, as well as those of the HTLV-I group, end within 50 amino acids downstream of the TM domain. Most lentivirus TM proteins, on the other hand, are considerably longer and may extend for more than 100 residues beyond the TM domain. In EIAV, this C-terminal region contains more than 200 amino acids. The difference in length between the lentivirus TM proteins and those of other retroviruses suggests a lentivirus-specific function for the long C-terminal tail. As an initial step toward defining a role for this protein segment in the viral life cycle and/or pathogenesis, we studied the synthesis and processing of the EIAV TM protein.

MATERIALS AND METHODS

Cells and virus. EIAV-producing cultures of equine fetal kidney cells were grown in Eagle minimal essential medium containing 15% fetal calf serum, 1% sodium pyruvate, and nonessential amino acids. Uninfected and EIAV-infected canine thymus (Cf2Th) cells were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum. Cultures of horse macrophages were established by seeding 6×10^8 buffy coat cells from fresh blood into a T150 plastic tissue culture flask with 30 ml of RPMI 1640 containing 20% fetal calf serum, 30% calf serum, and 1% sodium pyruvate. Nonadherent cells were removed after 2 h, and Wyoming strain virus was applied in the amount of 1.5 ml of viral stock (containing $10^{4.8}$ horse leukocyte infectious doses per ml) per T150 flask. Cultures were maintained for up to 11 days, by which time the infected cultures, but not the uninfected controls, showed a marked cytopathic effect indicative of EIAV infection. To isolate the virus, culture supernatants were centrifuged at $200 \times g$ for 4 min to remove cells and cell debris, and the resulting supernatant was centrifuged at $100,000 \times g$ for 90 min to pellet the virus.

Metabolic labeling. Cells were washed once in phosphate-buffered saline and incubated for about 10 min at 37°C in medium containing 10% dialyzed fetal calf serum but lacking

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methionine and cysteine. [^{35}S]methionine and [^{35}S]cysteine (600 to 1,000 Ci/mmol; Amersham Corp.) were added to a final level for each of 50 to 100 μCi/ml. For analysis of cellular lysates, the incubation time was 2 h; for analysis of viral particles, the incubation time was 5 h. Virus was isolated as described above for the macrophage cultures.

Immune precipitation. Viral pellets or cells were lysed in TNT buffer (20 mM Tris [pH 7.5], 200 mM NaCl, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride and 200 Kallikrein units of aprotinin per ml, and lysates were centrifuged for 10 min at 10,000 × *g* in an Eppendorf microfuge. Viral supernatants were incubated directly with antisera and protein A-Sepharose (Pharmacia, Inc.). Cellular supernatants were first boiled in 1% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol (βME) before dilution with TNT and incubation with antisera and protein A-Sepharose. Immune precipitates were collected, washed in TNT, boiled in gel loading dye, and applied to SDS-polyacrylamide gels. For endoglycosidase F (endo F) digestion, a 30 μl sample of a solution containing 0.05 M potassium phosphate (pH 6.8), 20 mM EDTA, 1% βME, 1% Triton X-100, and 0.2% SDS was added to the washed immune precipitates; the suspensions were boiled for 5 min; and 0.2 U of endo F (grade II; Boehringer Mannheim Biochemicals) was then added to each sample. They were incubated at 37°C for 4 h, whereupon they were boiled in gel loading dye and applied to a gel.

Immunoblotting. The virus-containing pellet from an 8-day culture of EIAV-infected horse macrophages was suspended in 100 μl of gel loading dye and boiled. The high-speed pellet from a mock-infected culture was treated identically. Samples of 15 μl per lane were fractionated on a 10- to 20% SDS-polyacrylamide gel. After electrophoresis, the gel was electroblotted onto an Immobilon membrane filter (Millipore Corp.), and filter strips were subsequently incubated with 1:100 dilutions of antisera and then with ^{125}I-protein A at 3 × 10^5 cpm/ml. Purified virus from equine kidney cells and from canine thymus cells was also analyzed; each lane contained about 25 μg of virus.

Infections and electroporation. A total of 10^5 uninfected Cf2Th cells in a 60-mm (diameter) dish was incubated for 30 min in complete medium containing 20 μg of DEAE-dextran per ml. This medium was then removed, and 0.5 ml of virus-containing medium was applied and incubated for 30 min. Complete medium containing 5 μg of Polybrene per ml was then added. Cultures were propagated and analyzed for virus production after several passes. The virus-containing medium was taken from a confluent virus-producing culture and was clarified and sterile filtered before use. For electroporation, 50 μg of DNA prepared as described previously (35) was added to 10^6 Cf2Th cells in 1 ml of ice-cold phosphate-buffered saline. The mixture was pulsed, allowed to rest 10 min at 0°C, and diluted with 1 ml of complete medium. After 10 min at room temperature, the mixture was transferred to a T75 culture flask and propagated as usual.

Amplification of a portion of the *env* gene. Deoxyoligonucleotides used in the amplification were 5′-GGATTCCTG GATTGGGAGC-3′ (bases 509 to 527 of the TM-protein-coding sequence) and 5′-CAATTGTCAGAATACAAGC AC-3′ (complementary to bases 168 to 188 of the long terminal repeat). A 0.5-μg portion of cellular DNA was mixed with 20 pmol of each oligonucleotide in 100 μl of buffer containing 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each all four deoxynucleoside triphosphates, and 2.5 U of *Thermus aquaticus* polymerase (Cetus-Perkin Elmer). The resulting mixture was subjected to 35 cycles of denaturation (95°C for 1 min), annealing (50°C for 2 min), and extension (72°C for 10 min), by using the Ericomp thermal cycler. Amplification of a 0.96-kilobase (kb) fragment was confirmed by electrophoresis of a sample of the product. The remainder of the reaction product was extracted with an equal volume of chloroform, and DNA was precipitated by the addition of 2 volumes of ethanol. The suspended precipitate was digested with the restriction endonucleases *Apa*I and *Spe*I, yielding a 0.47-kb fragment, which was purified by electrophoresis in a 1.2% gel of low-melting-point agarose. The *Apa*I and *Spe*I sites follow bases 603 and 1071, respectively, of the TM-protein-coding sequence. This fragment was cloned into BlueScript-KS (Stratagene), which had been digested with *Apa*I and *Spe*I. Individual white colonies were selected and expanded, and minipreparations of DNA were sequenced by the dideoxy-chain termination method (37) by using the Sequenase system and protocol (U.S. Biochemical Corp.). The sequencing primer was 5′-GGCCCTCTGGAAAGTG ACC-3′ (bases 600 to 618 for the TM-protein-coding sequence).

Peptides and antisera. Three TM protein peptides were synthesized, coupled, and administered to rabbits as described previously (34). The sequence of peptide tm-1 is Lys-Glu-Arg-Gln-Gln-Val-Glu-Glu-Thr-Phe-Asn-Leu-Ile-Gly-Cys (residues 77 to 91 of the TM protein [2, 36]), and it was coupled to keyhole limpet hemocyanin via its C-terminal cysteine. Peptide tm-2 is Cys-Gly-Ser-Gly-Asp-Lys-Tyr-Tyr-Lys-Gln-Lys-Tyr-Ser-Arg (residues 247 to 259 of the TM protein), and peptide tm-3 is Cys-Leu-Asn-Pro-Gly-Thr-Ser-His-Val-Ser-Met-Pro-Gln-Tyr-Val (residues 402 to 415). The N-terminal cysteines do not occur in the EIAV sequence and were added to facilitate coupling.

Surface glycoprotein peptides were purchased in crude form from Peninsula Laboratories. Peptide sgp-1 is Cys-Ala-Glu-Ser-Lys-Glu-Ala-Arg-Asp-Gln-Glu-Met-Asn-Leu-Lys-Glu (residues 41 to 55 of the mature protein [1, 29]), and peptide sgp-2 is Cys-Leu-Pro-Ile-Ser-Ser-Glu-Ala-Asn-Thr-Gly-Leu-Ile-Arg-His-Lys-Arg (residues 424 to 439). The N-terminal cysteines of each were added to facilitate coupling.

RESULTS

To investigate the EIAV *env* gene products, antisera to five *env* peptides were raised (Fig. 1). Two of the peptides are part of the surface glycoprotein (one near the N-terminus and the other at the extreme C-terminus), and three are part of the TM protein (one upstream of the putative membrane-spanning region and two in the long C-terminal extension). Rabbit antisera to these peptides were used in immune precipitation and immunoblotting experiments with viral and cellular lysates.

Identification of *env* gene products by immune precipitation. Immune precipitation of radiolabeled viral proteins with the surface glycoprotein antisera (anti-sgp-1 and anti-sgp-2) revealed in SDS-polyacrylamide gel electrophoresis (PAGE) analysis a single broad band corresponding to gp90, as described by Parekh et al. (33). The protein differs somewhat in size in virus produced by cultured equine fetal kidney cells (100 kilodaltons [kDa]; Fig. 2, lanes 4 and 9) and in virus produced by EIAV-infected canine thymus cells (90 kDa; lanes 2 and 7). Removal of carbohydrate side chains with endo F resulted in the disappearance of the 90- and 100-kDa species and the appearance (in both cases) of a 48-kDa protein (lanes 3, 5, 8, and 10). This size agrees very well with that predicted for the unglycosylated surface glycoprotein by

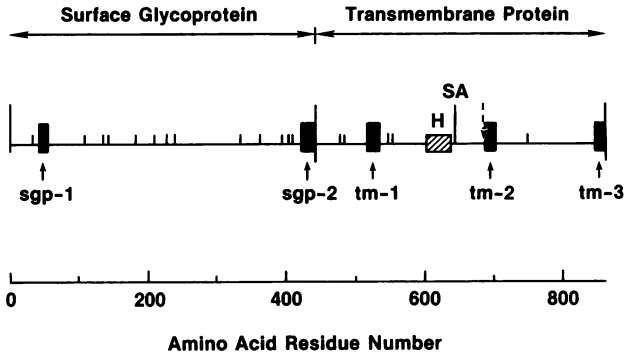

FIG. 1. *env* gene of EIAV. N-termini of the mature surface glycoprotein and of the TM protein have been determined by Ball et al. (2). Short vertical bars represent potential N-linked glycosylation sites. Black boxes represent peptides against which rabbit antisera were raised. The dashed arrow immediately upstream of the tm-2 peptide indicates the cleavage site determined in this study. Abbreviations: H, putative membrane-spanning region; SA, splice acceptor found in *tat-rev* mRNA (8, 38a).

the nucleotide sequence (439 amino acids) (23, 36). Since the 48-kDa protein was produced by virus both from canine and equine cells, the difference in the mature forms must result from their pattern and/or extent of glycosylation and sialylation. Thus, antisera to peptides near the N- and C-termini of the predicted surface glycoprotein revealed a single protein species of the expected molecular weight.

In contrast, antisera to peptides from the predicted TM protein recognized more than one protein in lysates of radiolabeled particles produced by cultured canine cells. Whereas the TM sequence encodes 415 amino acids, antiserum to peptide tm-1 precipitated a species of average size,

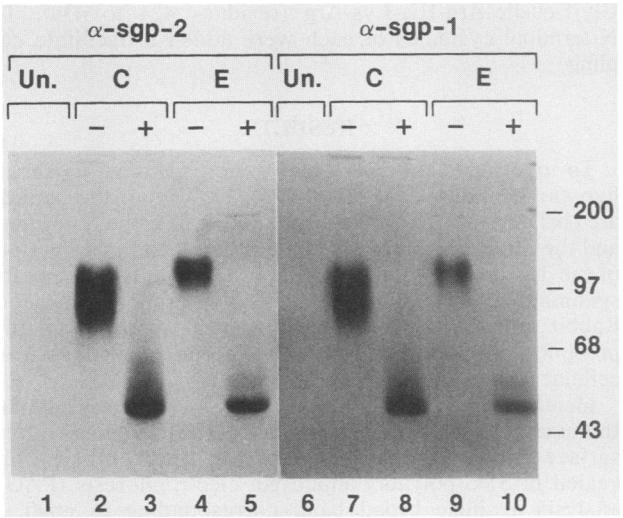

FIG. 2. Precipitation of the EIAV surface glycoprotein. Virus-producing canine thymus cells (C) and equine kidney cells (E) as well as uninfected canine cells (Un) were grown in [^{35}S]methionine and [^{35}S]cysteine for 5 h. Virus was isolated by high-speed centrifugation of the medium, and viral lysates were immune precipitated with the indicated antiserum. Half of each sample from the infected cells was then treated with endo F (+); the second half was untreated (−). Samples were analyzed by SDS-PAGE. Molecular masses (kilodaltons) of marker proteins are indicated to the right of the gel.

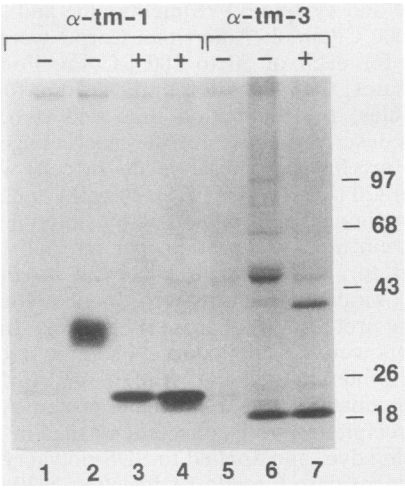

FIG. 3. Precipitation of viral lysates with TM protein antisera. Virus-producing canine cells were labeled, virus was harvested, and viral lysates were immune precipitated and treated with endo F (+) or untreated (−), as described in the legend to Fig. 2. Lanes 1 and 5, Immune precipitation was carried out in the presence of excess competing peptide tm-1 or tm-3, respectively. Lane 4, Whole disrupted virus was treated with endo F, followed by immune precipitation with anti-tm-1. Molecular masses (kilodaltons) of marker proteins are indicated to the right of the gel.

only 32 kDa (Fig. 3, lane 2). Upon endo F treatment, the size of this molecule was reduced to about 23 kDa (lane 3), which is clearly too small to represent the entire coding region. Antiserum to the C-terminal peptide tm-3, on the other hand, did not precipitate the 32-kDa species but instead recognized an unglycosylated molecule of about 20 kDa (lanes 6 and 7). The simplest interpretation of these results is that the 415-residue TM protein undergoes cleavage to an upstream gp32 portion and a downstream p20 segment. The 46-kDa protein in lane 6 probably represents the uncleaved full-length molecule, which, upon endo F treatment, was reduced to 40 kDa (lane 7). This interpretation is tentative, however, since the putative full-length protein was not recognized by anti-tm-1 (lane 2).

Essentially the same results were obtained with virus from equine kidney cells. As with the surface glycoproteins (gp90 and gp100), the upstream portion of the TM protein was slightly larger in the equine cells (gp35) than in the canine cells (gp32) (Fig. 4A, lanes 2 and 4), but endo F treatment resulted in a 23-kDa protein in both cases (Fig. 4A, lanes 3 and 5). In addition, virus from the canine cells contained (after digestion) a 22-kDa protein (C in Fig. 4A, lane 3), the origin of which is documented below. As in the canine cells, anti-tm-3 precipitated an unglycosylated p20 as well as the putative full-length uncleaved TM protein (Fig. 4B, lanes 4 and 5). None of these proteins was detectable in uninfected cells (Fig. 4A and B, lanes 1).

To confirm a viral origin for the observed proteins, we used four additional antisera. Each of three antisera from EIAV-infected horses precipitated not only the surface glycoprotein but also gp32 and gp35 (Fig. 4A, lanes 7, 9, 11, and 12). Like anti-tm-3, anti-tm-2 precipitated p20 from canine and equine cells (Fig. 4B, lanes 7 and 8) but not from uninfected cells (Fig. 4B, lane 6). Anti-tm-2 also recognized the putative uncleaved gp48; its size, like those of gp90 and gp100 and gp32 and gp35, appears to differ slightly between the canine and equine cell viruses (Fig. 4B, lanes 2, 4, 7, and

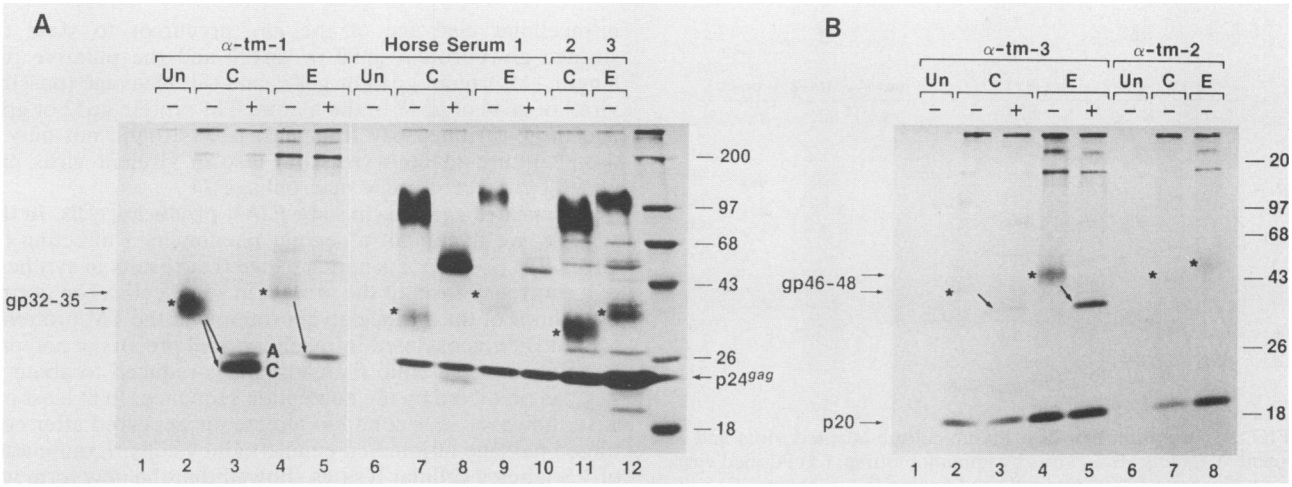

FIG. 4. gp35 and p20 in virus from canine and equine cells. Virus-producing canine cells (C) and equine kidney cells (E), as well as uninfected canine cells (Un), were labeled, and virus was harvested and treated as described in the legend to Fig. 2. After immune precipitation, samples were treated with endo F (+) or untreated (−). Horse sera 2 and 3 are from asymptomatic carriers of EIAV; horse serum 1 is from an acutely infected horse. Arrows point to the mature TM proteins gp32 and gp35 (*) and to $p24^{gag}$ (panel A) and to the putative full-length TM proteins gp46 and gp48 (*) and to p20 (panel B). Bands A and C in panel A, lane 3, are the mature TM protein and a shortened form of the TM protein, respectively. Molecular masses (kilodaltons) of marker proteins are indicated to the right of each gel.

8). Thus, results with three peptide antisera and three horse sera indicate that the EIAV TM protein exists in two fragments in the virus, an upstream gp32 or gp35 and a downstream p20.

Isolation and N-terminal sequencing of p20. The isolation and purification by reversed-phase high-pressure liquid chromatography of EIAV *gag* proteins have been described previously (18). Briefly, disrupted virus was fractionated in an acetonitrile gradient and the fractions were assayed by SDS-PAGE. Major peaks of optical density were analyzed for the N-terminal sequence, and the viral origin of the protein in question was confirmed by reference to the nucleotide sequence (38). During the course of this work, a protein of about 20 kDa was found and shown to have the N-terminal sequence Leu-Ala-Gly-Val-Thr-Gly-Gly-Ser-Gly-Asp-Lys-Tyr-Tyr. Subsequently, the nucleotide sequence of the EIAV *env* gene was reported (36). It showed that this protein begins 175 amino acids upstream of the C terminus of the predicted *env* precursor protein, making it a likely candidate for the p20 described above. This result is completely consistent with the immune precipitation experiments, which predict a cleavage site immediately upstream of peptide tm-2. In the sequence given above, the third glycine (underlined) is the N terminus of peptide tm-2, so that cleavage occurred only six residues upstream. We conclude that p20 predicted from the use of peptide antisera in precipitation experiments can be identified in bulk preparations of purified virus.

The actual cleavage site predicted from the amino acid sequence above together with the nucleotide sequence is Ile-His/Leu-Ala. While not typical of sequences cleaved by viral proteases, it is not so dissimilar that cleavage by a cellular enzyme must be invoked. In particular, there is precedent among the viral proteases for cleavage next to a His residue, and branched amino acids such as Ile are often observed at P2 and P2′ sites (21).

Cleavage does not occur intracellularly. Examination of cellular rather than viral lysates should reveal whether cleavage to gp32 or gp35 and p20 occurs intracellularly or after budding. In virus-producing equine kidney cells grown for 2 h in ^{35}S-labeled precursors, we expect to detect the newly synthesized envelope precursor (854 amino acids). Since cleavage of this precursor to yield the surface glycoprotein and the full-length TM protein is expected to be accomplished by a cellular enzyme (by analogy to other retroviruses), these two proteins should be detected as well. The question is will gp35 and p20 be seen? Figure 5 shows that they are not. Four *env* antisera precipitated the high-molecular-weight glycosylated translational product of the *env* gene, gPr^{env}, which, upon treatment with endo F, was reduced in size to about 95 kDa, as expected. Anti-sgp-2 also precipitated the mature surface glycoprotein gp100, which (as in Fig. 2, lane 5) was reduced to 48 kDa upon endo F treatment (Fig. 5, lanes 1 and 2). Anti-tm-2 and anti-tm-3 precipitated the 48-kDa endo F-sensitive protein we presume

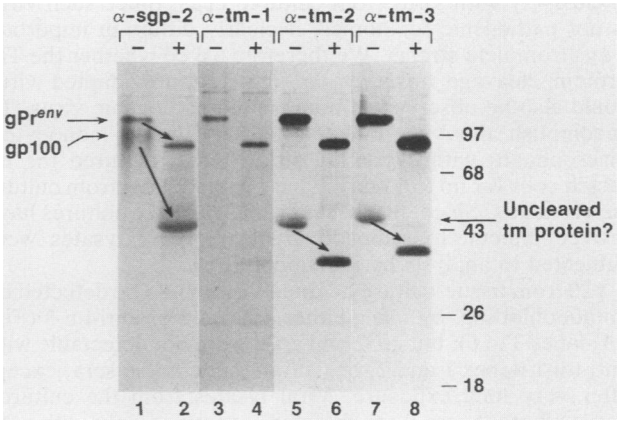

FIG. 5. gp35 and p20 are not detectable in lysates of infected cells. Virus-producing equine kidney cells were grown for 2 h in [^{35}S]methionine and [^{35}S]cysteine, lysed, boiled in 1% SDS and 1% βME, and immune precipitated with the indicated antisera. Half of each washed precipitate was treated with endo F (+); half was untreated (−). Samples were analyzed by SDS-PAGE. Samples in lanes 7 and 8 were analyzed on a different gel from those in lanes 1 to 6. gPr^{env}, Envelope precursor. Molecular masses (kilodaltons) of marker proteins are indicated on the right.

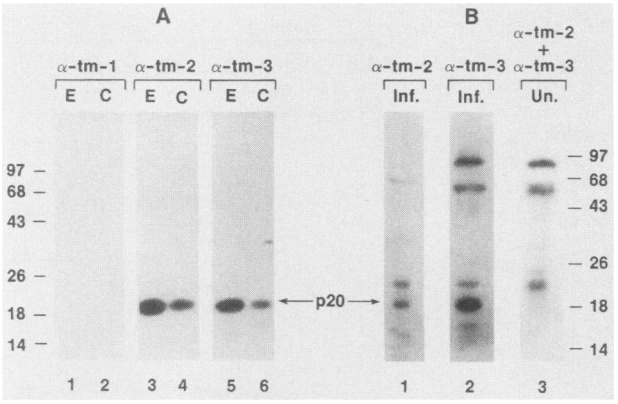

FIG. 6. Detection of p20 in tissue-culture-adapted virus and in virulent Wyoming strain virus by immunoblotting. (A) Purified virus (about 25 μg per lane) from cultured equine (E) or canine (C) cells was lysed, fractionated by SDS-PAGE, and blotted onto an Immobilon membrane filter. Filter strips were incubated with the indicated antiserum at a dilution of 1:100, washed, and then incubated with ^{125}I-protein A. (B) Primary cultures of horse monocyte-macrophages were infected (Inf) with Wyoming strain virus. Eight days after infection, the cell supernatant was collected and virus was pelleted by high-speed centrifugation. Viral lysates were treated as described for panel A. Un, Lysate of high-speed pellet from cell supernatant of uninfected cells. Molecular masses (kilodaltons) of marker proteins are indicated beside each gel.

to be the full-length TM protein (Fig. 5, lanes 5 to 8). However, neither anti-tm-1 (lane 3) nor natural horse serum detected gp35, and neither anti-tm-2 nor anti-tm-3 precipitated p20 (lanes 5 and 7). We conclude that the cleavage which gives rise to gp35 and p20 occurs later, perhaps in the virus. We have tried to verify a viral cleavage by pulse-chase experiments, but so little TM protein (either full length or cleaved) is found in virus that the amount of radioactive precursor needed for a very short labeling period is prohibitive.

Transmembrane protein cleavage also occurs in an EIAV field strain. The experiments reported above were performed exclusively with virus from cultured cells. Since such virus is not pathogenic for horses, it clearly differs in important ways from field strains. We therefore asked whether the TM protein cleavage observed in tissue-culture-adapted virus could also be observed in nonadapted pathogenic virus. To accomplish this, we infected primary horse monocytes-macrophages with Wyoming strain virus, cultured the infected cells for up to 11 days, and pelleted virus from culture supernatants. Since, in our hands, macrophage cultures have proved difficult to radioactively label, viral lysates were subjected to analysis by immunoblotting.

p20 from tissue-culture-adapted virus could be detected by immunoblotting, by using either anti-tm-2 or anti-tm-3 (Fig. 6A, lanes 3 to 6), but gp32 and gp35 were not detectable with anti-tm-1 (lanes 1 and 2) or with immune horse sera, except after very long exposure. Viral lysates from the cultured horse macrophages were therefore examined only with anti-tm-2 and anti-tm-3. Both of these antisera recognized a protein of 20 kDa that was not present in the uninfected control (Fig. 6B). We conclude that the cleavage to gp32 or gp35 and p20 is not a phenomenon restricted to nonpathogenic tissue-culture-adapted virus but occurs in Wyoming strain virus in its presumptive natural target cell as well.

In summary, synthesis of the EIAV TM protein proceeds in the following steps: (i) synthesis of the *env* precursor, (ii) intracellular cleavage of the *env* precursor to yield the surface glycoprotein gp90 or gp100 and the putative full-length TM protein gp46 or gp48, and (iii) cleavage (possibly viral) of gp46 or gp48 to the mature TM protein gp32 or gp35 and the C-terminal p20 fragment. p20 is found not only in tissue-culture-adapted virus but also in virulent virus produced by infected horse macrophages.

A truncated *env* gene in some EIAV-producing cells. In this section, we document a second phenomenon affecting the EIAV TM protein: a genetic change that results in synthesis of a shortened form of the protein in some cells. The normal progenitor of the surface glycoprotein and the TM protein is a 135-kDa glycosylated intracellular *env* precursor polyprotein which, upon endo F treatment, is reduced to about 95 kDa, as predicted by the nucleotide sequence. In at least one case, however, a second *env* precursor appeared after continued passage of infected equine kidney cells. Examination of ^{35}S-labeled cellular lysates showed that the new form was smaller (about 115 kDa) and was reduced to about 78 kDa after endo F treatment (Fig. 7A, lanes 6 to 9). Since the smaller precursor (Pr_2) was precipitated by both surface glycoprotein antisera and by anti-tm-1 and anti-tm-2 (lanes 1 to 4) but not by anti-tm-3 (lane 5), it appears to be truncated at its C terminus relative to the full-size precursor Pr_1. Since it lacks about 18 kDa relative to Pr_1 but was still recognized by anti-tm-2, the truncation must have occurred immediately downstream of peptide tm-2. Both forms of precursor were synthesized by these cells. Virus-producing canine thymus cells also synthesized two *env* precursors (Fig. 7B). In this case, however, truncation must have occurred upstream of peptide tm-2, since the shortened form is recognized by anti-tm-1 (lane 5) but not by anti-tm-2 or anti-tm-3 (lanes 8 and 11).

Virus expressing the truncated *env* precursor is enriched upon passage. We tested whether virus expressing the shortened *env* precursor is infectious. Virus from the parental canine cell culture (which expresses both Pr_1 and Pr_2) was applied to uninfected canine cells (generating culture II [Fig. 8A]). In addition, cellular DNA from the parental culture was introduced into uninfected cells by electroporation (generating culture I). After several weeks of passaging, cultures I and II were grown in ^{35}S-labeled amino acids, and lysates were examined by immune precipitation. Both cultures contained readily detectable truncated precursor (Pr_2) but little or no full-size precursor (Pr_1) (Fig. 8B, lanes 2 and 4). Thus, upon passage to new cells, a significant enrichment for the truncated *env* form occurred.

The enrichment process continued through a second round. Virus from culture II was added to uninfected cells, generating culture III, and DNA from culture II was introduced into uninfected cells by electroporation, generating culture IV. Subsequent immune precipitation revealed very little Pr_1 in culture III and no detectable Pr_1 in culture IV (Fig. 8B, lanes 6, 8, and 12). All of the *env* precursor in culture IV was the same size as Pr_2 of the parental culture (lanes 7 to 10).

Two conclusions can be drawn from these results. First, since the electroporated cultures I and IV contained only Pr_2, virus expressing only Pr_2 must be infectious for canine cells. Second, since passage of virus from the parental culture to culture II and from culture II to culture III led to enrichment of Pr_2, virus expressing Pr_2 must be more infectious than virus expressing Pr_1 and/or cells infected with the Pr_2 virus must have a growth advantage over those infected with Pr_1 virus.

Truncation is due to a genetic change. We hypothesized

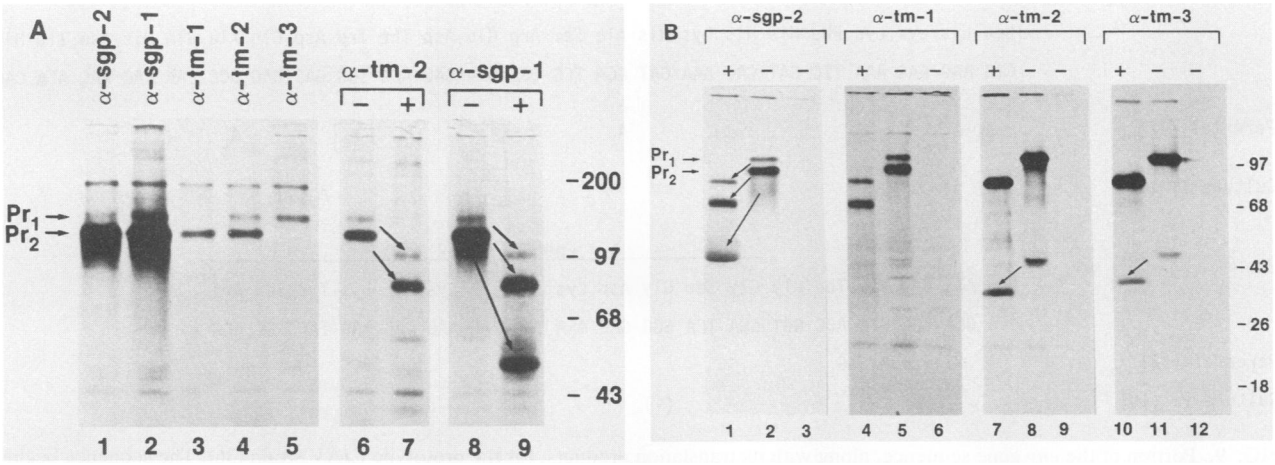

FIG. 7. Two sizes of envelope precursor protein. (A) Equine kidney cells. (B) Canine thymus cells. Virus-producing cells were grown in ^{35}S-labeled amino acids for 2 h, lysed, boiled in 1% SDS and 1% βME, and immune precipitated with the indicated antiserum. Lanes: +, samples treated with endo F after precipitation; −, untreated. Pr_1 and Pr_2 are the full-size and truncated precursors, respectively. Samples in panel A, lanes 6 to 9, were not boiled before precipitation; samples in panel B, lanes 3, 6, 9, and 12, were uninfected; samples in lanes 10 to 12 were analyzed on a different gel from those in lanes 1 to 9. Molecular masses (kilodaltons) of marker proteins are indicated to the right of each panel.

that Pr_2 resulted from a mutant genome(s) containing either a 3′ deletion or a premature translation termination signal in the *env* gene. Since Pr_2 was recognized by anti-tm-1 but not by anti-tm-2 or anti-tm-3 in the canine cells, such a mutation presumably occurred upstream of the tm-2 peptide. To test this theory, we amplified, subcloned, and sequenced a portion of the *env* gene from the parental Cf2Th(EIAV) culture and from culture II.

The sequences of 12 *env* subclones from the parental culture revealed that four of them had in-frame termination codons slightly upstream of peptide tm-2 (Fig. 9). Single-base changes (relative to the prototype sequence [36]) at 11 other positions were also observed among the subclones, but these did not result in termination signals. Thus, an open reading frame was maintained in eight of the subclones. These results are consistent with the synthesis of both Pr_1 and Pr_2 by cells of the parental culture.

In contrast, 8 of 10 subclones from culture II had in-frame terminators upstream of peptide tm-2. One additional subclone terminated downstream of peptide tm-2 as a result of a single-base deletion. Only 1 of the 10 maintained an open frame in this region. These results account for the preponderance of Pr_2 synthesized by culture II. Thus, the truncated *env* precursor results from premature termination codons in the *env* gene. In 12 of the 13 cases we observed, these terminators were located 7, 9, or 12 codons upstream of the gp32-p20 cleavage site.

A truncated TM protein is detectable in virus. Cleavage products expected from the full-size and truncated precursors are shown in Fig. 10. Cleavage of Pr_1 yields the surface glycoprotein, the mature TM protein (gp32-35), and p20, as documented in the preceding sections of this report. Cleavage of equine Pr_2, which extends through peptide tm-2, should also yield the mature TM protein (A in Fig. 10) as well

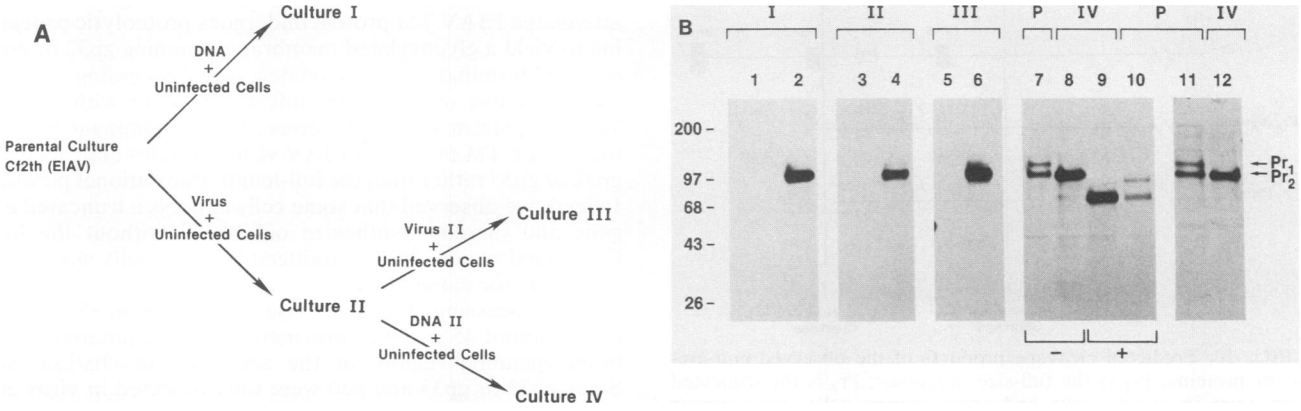

FIG. 8. Viral passage results in enrichment of the truncated precursor form. (A) Generation of new cultures of canine cells expressing EIAV. (B) Immune precipitation of envelope precursors in the new cultures. The parental culture (P) and cultures I to IV were grown in ^{35}S-labeled amino acids, lysed, and immune precipitated with anti-tm-1 (lanes 1 to 10) or horse serum with antibodies to EIAV (lanes 11 and 12). Lanes 1, 3, and 5, Precipitation carried out in the presence of excess peptide tm-1; +, endo F-treated after immune precipitation. Pr_1 and Pr_2 are the full-length and truncated envelope precursors, respectively. Molecular masses (kilodaltons) of marker proteins are indicated to the left of panel B.

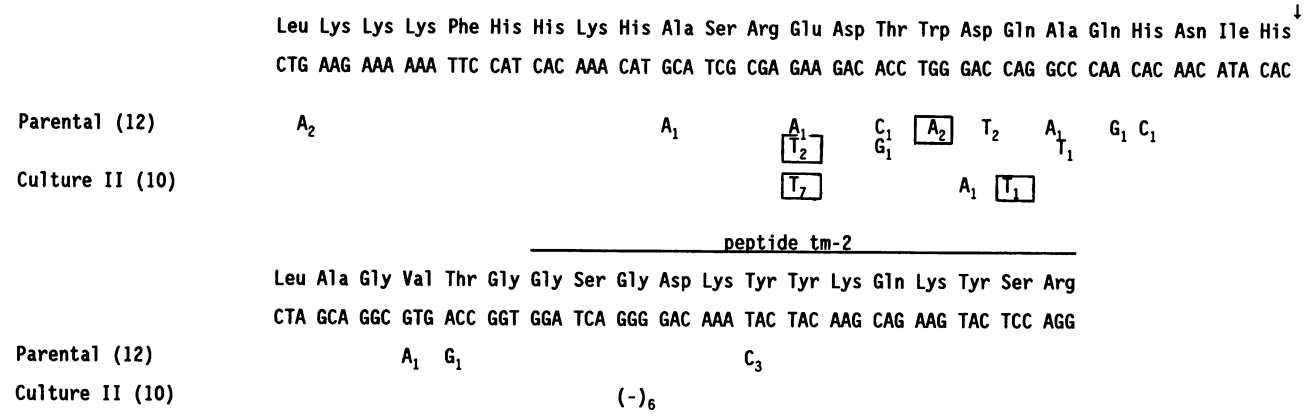

FIG. 9. Portion of the *env* gene sequence, along with its translation products, for the prototype EIAV strain (36). The sequence begins 20 residues downstream of the putative membrane-spanning region of the TM protein and continues through the peptide tm-2 region (residues 217 to 259 of the TM protein). Cellular DNAs from the parental Cf2Th(EIAV) culture and from culture II were amplified by using the polymerase chain reaction, and amplified material was subcloned and sequenced. The number of subclones that were sequenced is given in parentheses. Sequences were identical to the prototype except where indicated. Subscripts denote the number of clones with each change. Boxes indicate that stop codons were created by the sequence changes. The dash denotes a deletion. The arrow indicates the cleavage site between gp32 and p20.

as a short peptide (B in Fig. 10) that would not be detected in immune precipitation experiments. Thus, no new or altered products are expected in virus produced by equine cells expressing both precursor forms relative to those expressing only Pr_1. In virus produced by the parental canine culture, however, where Pr_2 terminates 7 to 9 codons upstream of the gp32-p20 cleavage site, a shortened form of TM protein (C in Fig. 10) is expected. In fact, endo F digestion of gp32 revealed a 22-kDa protein (C in Fig. 4, lanes 3 and 8) in addition to the normal 23-kDa mature TM protein (Fig. 4A). Thus, absence of the long C-terminal fragment did not prevent incorporation of the truncated TM protein into virus particles, and, as shown above, the resulting virus is infectious.

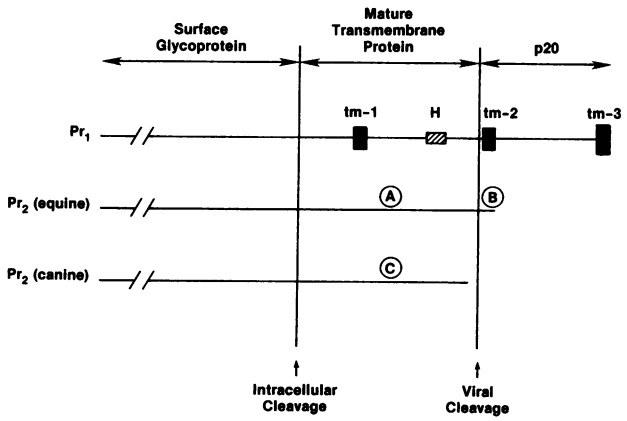

FIG. 10. Predicted cleavage products of the observed *env* precursor proteins. Pr_1 is the full-size precursor; Pr_2 is the truncated form seen in canine cells and some equine cells; presumptive cleavage products of Pr_2 include the mature TM protein (A) and two short peptides (B and C). tm-1, tm-2, and tm-3 are peptides against which antisera were raised. H, Very hydrophobic region that is the presumptive membrane-spanning segment. Cleavage at the C terminus of the surface glycoprotein is intracellular; cleavage at the C terminus of the mature TM protein may be viral.

DISCUSSION

In general, EIAV strains capable of causing disease in horses cannot be propagated in established tissue culture lines and will only replicate well in primary horse macrophages (24, 26). This is consistent with the observation that the primary targets in vivo are cells of the monocyte-macrophage lineage (24, 30, 31, 35). The commonly used Malmquist variant of the virulent Wyoming strain was established only after extended passaging in macrophages followed by successful infection of equine dermal or kidney cells (27, 29). This variant could subsequently be passed in a variety of different equine cell lines as well as in feline and canine cells (3), but it does not cause disease in horses. Proviral DNA clones of this attenuated virus have been completely sequenced (23, 36, 38), and *gag and env* proteins have been isolated and partially or completely sequenced (2, 18). It is primarily this attenuated virus that has been examined in the present study.

Our first finding is that most (and perhaps all) of the attenuated EIAV TM protein undergoes proteolytic processing to yield a glycosylated membrane-spanning gp32 or gp35 and a C-terminal nonglycosylated p20. Processing also occurs in equine macrophages infected in vitro with virulent Wyoming strain virus. Therefore, the predominant form of the mature TM protein in EIAV virus particles appears to be gp32 or gp35 rather than the full-length translational product. Indeed, we observed that some cells express a truncated *env* gene and thereby synthesize only gp32 without the long C-terminal region. Virus produced by such cells was highly infectious for canine cells.

The processing that gives rise to gp32 or gp35 and p20 occurs about 45 residues downstream of the putative membrane-spanning region, at the sequence Ile-His/Leu-Ala. Since gp32 or gp35 and p20 were only detected in virus and not intracellularly, it is possible that processing is carried out by the viral protease. The sequence at the cleavage site is not inconsistent with this possibility, and we are currently testing whether purified EIAV protease will cleave a synthetic peptide containing this sequence.

In HIV-1, most of the TM protein appears to be unproc-

essed (41), though a small amount of a C-terminal peptide was found in virus fractionated by high-pressure liquid chromatography (17). C-terminal processing of other lentivirus TM proteins has not been reported, but there are precedents among the much shorter TM proteins in the murine family of the type C retroviruses (13, 39). As with EIAV, the role of this cleavage in the viral life cycle is unknown and awaits mutagenesis experiments with infectious molecular clones.

Our second finding is that some cells fail to express p20 at all. In two different cell lines expressing attenuated virus, we observed truncated forms of the envelope precursor polyprotein. The point of truncation differed in the two instances, but both occurred just slightly downstream of the putative membrane-spanning region of the TM protein. In the case of the equine fetal kidney cell line, the genome expressing the truncated form was not infectious for equine dermal cells. The reason for the lack of infectivity is unknown but may be unrelated to a shortened envelope protein (for example, truncation might have resulted from an insertion or deletion, in which case the presumptive *rev* gene product would also be altered). In the canine thymus cell line, however, virus expressing the truncated form appeared to be much more infectious for canine cells than was virus expressing the full-size precursor. Infection of new cells, whether by exposure to virus-containing culture media or by electroporation of DNA, resulted in progressive enrichment of the short form.

In the absence of an infectious molecular clone of EIAV, we are unable to test whether the truncated envelope protein and increased infectivity are causally related. However, these results are very reminiscent of those seen with the simian immunodeficiency virus (SIV). When propagated in either cultured macaque lymphocytes or a rhesus monkey, the TM protein of SIV_{mac} has been found to be the full-length 41-kDa form (19, 25, 32). When propagated in human cells, however, rapid selection for a truncated form occurred (4, 5, 19, 25, 32, 42). Truncation resulted from the appearance of an in-frame termination codon immediately downstream of the membrane-spanning region (4, 5, 9, 16, 19, 20, 32). When virus expressing both the full-size and the truncated envelope was introduced into macaque lymphocytes or into a monkey, rapid selection for the full-size form occurred (19, 25). Thus, depending on the host cell, the SIV (and presumably EIAV) C-terminal extension of the TM protein plays different roles. In some cells, it is not required and even inhibits virus spread; but in cells of the animal in which the virus causes disease, it is advantageous. Termination codons in the TM-protein-coding sequence have also been found in some but not all isolates of HIV-2 (14, 28, 44) and SIV_{agm} (1, 10, 43).

The role of this region of the lentivirus in TM protein is unknown. Indeed, we do not even know whether it plays a role inside the infected cell during glycoprotein processing and transport or during virus assembly, at the outside of an uninfected cell during virus entry, or both. One approach to this problem is to determine the location of p20 relative to the viral membrane. There is a potential glycosylation site midway through p20 that remains unmodified (endo F had no effect on the size of the p20 fragment). This suggests that at least the midregion of the molecule was confined to the cytoplasmic side of the endoplasmic reticulum and hence is inside the virus. Similarly, immune horse antisera do not recognize p20-derived peptides (R. Montelaro, personal communication), perhaps suggesting that p20 does not appear on the cell or viral surface. However, recent studies of HIV-1 have suggested that its C-terminal region is at least membrane associated (15) and possibly membrane spanning (40). Thus, at least part of the region may be on the outside of the virus (20, 40). R. Montelaro (personal communication) has pointed out that the EIAV C-terminal region contains structural features (such as an amphipathic helix) that resemble those of HIV-1 (15, 40), suggesting that the orientation of the EIAV TM protein is similar to that of HIV-1. Use of the TM peptide antisera described in this report may help us determine the locations of the various regions of the EIAV protein.

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